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STRUCTURAL AND FUNCTIONAL ANALYSIS OF WNT RECEPTORS - WITH EMPHASIS ON FZD_6

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STRUCTURAL AND FUNCTIONAL ANALYSIS OF WNT RECEPTORS - WITH EMPHASIS ON FZD_6

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The more I learn, the more I learn how little I know. -- Socrates

ABSTRACT

Many cellular processes are dependent on the activation of G protein-coupled receptors (GPCRs). The development of drugs that can specifically target GPCRs and their corresponding signalling cascades is of high interest. Today, almost 1000 different GPCRs are known and even though about 40 % of all prescribed pharmaceuticals on the market target GPCRs either directly or indirectly, only a small fraction of receptors are druggable.

One specific Class of GPCRs are Frizzled (FZD), which belong to the Class F of GPCRs. Nineteen mammalian WNTs can bind to 10 FZDs in various combinations resulting in the activation of different downstream pathways such as WNT/β-catenin, WNT/planar cell polarity and WNT/Ca^{2+}. These signaling pathways are highly involved in cell polarity, embryonic development, formation of neural synapses, cell proliferation, differentiation and many other processes in developing and adult organisms. Mutations and/or misregulation within the pathways are linked to many diseases ranging from cancer, inflammatory diseases to metabolic and neurological disorders. Therefore, targeting WNT receptors pharmacologically would be advantageous. Currently very little is known in regard to structure and precise function of Class Frizzled receptors. Particular the link between FZDs and heterotrimeric G proteins is a matter of discussion in the field. Furthermore, the lack of high throughput screening assays hampers the development of small compounds targeting FZDs. This thesis provides advanced insight into structural and functional aspects of WNT receptors with the focus on FZD_6, from which not only future research but also drug discovery could benefit from. In addition, this thesis intends to provide novel tools to study this unconventinal class of receptors, which will allow extending the knowledge to the entire Class F and possible to other GPCRs.

LIST OF SCIENTIFIC PAPERS

I. **Disheveled regulates precoupling of heterotrimeric G proteins to Frizzled 6.**

Kilander MB*, **Petersen J***, Andressen KW, Ganji RS, Levy FO, Schuster J, Dahl N, Bryja V, Schulte G FASEB J. 2014 May;28(5):2293-305

II. **Agonist-induced dimer dissociation as a macromolecular step in G protein-coupled receptor signaling**

Julian Petersen*, Shane Wright*, David Rodríguez, Noa Lahav, Aviv Vromen, Johan Strömqvist, Stefan Wennmalm, Assaf Friedler, Jens Carlsson and Gunnar Schulte *manuscript*

III. **Identification of a molecular switch in TM6/7 in Frizzled 6 regulating receptor activation**

Julian Petersen*, Belma Hot*, Kateřina Straková, Jana Valnohová, Evgeny Ivashkin, Maria Consuelo Alonso Canizal, Carsten Hoffmann, Igor Adameyko, Vita Bryja, David Rodríguez, Jens Carlsson and Gunnar Schulte *manuscript*

IV. **Asymmetry of VANGL2 in migrating lymphocytes as a tool to monitor activity of the mammalian WNT/planar cell polarity pathway**

Markéta Kaucká*, **Julian Petersen***, Pavlína Janovská, Tomasz Radaszkiewicz, Lucie Smyčková, Avais M Daulat, Jean-Paul Borg, Gunnar Schulte, Vitezslav Bryja *Cell Communication and Signaling* (2015) 13:2

* These authors contributed equally.

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

Related published papers and database entry involving the author, which are not included in the thesis:

I. **WNT stimulation dissociates a Frizzled 4 inactive state complex with Gα12/13**
   Elisa Arthofer, **Julian Petersen**, Belma Hot, Kateřina Straková, J. Silvio Gutkind, Gunnar Schulte *manuscript*

II. **The Concise Guide to PHARMACOLOGY 2013/14: overview CGTP Collaborator**
   British Journal of Pharmacology (2013) 170(8):1449-58.

III. **WNT/Frizzled signaling: receptor-ligand selectivity with focus on FZD-G protein signaling and its physiological relevance: IUPHAR Review 3**
   Dijksterhuis JP*, **Petersen J***, Schulte G
   British Journal of Pharmacology (2014) 171(5):1195-209.

IV. **Frizzled Class GPCRs entry on the IUPHAR database**
   Contributor
   http://www.guidetopharmacology.org/GRAC/FamilyIntroductionForward?familyId=25

* These authors contributed equally.

CONTENTS

LIST OF ABBREVIATIONS

7TMD	seven transmembrane domain
aa	amino acid
Arg	arginine
B2AR	beta-2 adrenergic receptor
CD	circular dichroism
CETSA	cellular thermal shift assay
CK1	casein Kinase 1
CLL	chronic lymphocytic leukemia
CRD	cysteine-rich domain
Cys	cysteine
dcFRAP	dual-color fluorescence recovery after photobleaching
DEP	dishevelled, eg-10 and pleckstrin
DIX	dishevelled and axin
DVL	dishevelled
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinases
FCCS	fluorescence cross-correlation spectroscopy
FCS	fluorescence correlation spectroscopy
FRAP	fluorescence recovery after photobleaching
FRET	förster resonance energy transfer
FZD	frizzled
GDP	guanosine diphosphate
GEF	guanine nucleotide Exchange Factor
Glu	glutamate
GPCR	G protein–coupled receptor
GRK	G protein-coupled receptor kinase
GTP	guanosine triphosphate
HTS	high throughput screening
IC	intracellular

ITC	Isothermal titration calorimetry
IUPHAR	the international union of basic and clinical pharmacology
LRP5/6	low-density lipoprotein-related receptors 5 and 6
Lys	lysine
MAPK	mitogen-activated protein kinase
PCP	planar cell polarity
PDZ	Psd-95/Disc large/ZO-1 homologous
P-ERK1/2	phosphorylated ERK1/2
PM	plasma membrane
RGS	regulators of G protein signaling
ROR1/2	receptor tyrosine kinase-like orphan receptor 1 and 2
RYK	receptor-like tyrosine kinase
SFRP	secreted frizzled-related protein
SMO	smoothened
TCF	T-cell factor
Thr	threonine
TM	transmembane
TSH receptor	thyrotropin receptor
Tyr	tyrosine
UV-Vis	ultraviolet-visible spectrophotometry
Vangle1/2	van gogh-like protein 1 and 2
WNT	wingless/int-1
wt	wild type

1 INTRODUCTION

Cells are spatially defined by a plasma membrane that serves as a border between the cell interior and the surrounding environment. Exterior signals are detected by receptors embedded in the cell membrane and the encoding information is transferred into the cell interior. Such signal transduction can be accomplished in various ways. The majority of receptors for hormones and neurotransmitters mediate the signal to effector proteins located inside of the cell via the activation of heterotrimeric GTP-binding proteins (Gilman 1987, Dijksterhuis, Petersen et al. 2014). The superfamily of G protein-coupled receptors (GPCR) belongs to the largest protein-coding gene family in humans (McPherson, Marra et al. 2001). Almost 1000 various GPCRs have been identified to date. Their ligands range from biogenic amines, peptides, glycoproteins, lipids, nucleotides, to ions and proteases. GPCRs thus participate in innumerable physiological functions and even sensing exogenous stimuli such as taste, smell and light perception are mediated by this receptor family. GPCRs are divided into different classes based on their sequence homology and functional similarity. The first classification scheme that has been proposed for GPCRs divided them, on the basic of sequence homology, into six classes:

Class A (rhodopsin-like), Class B (secretin receptor family), Class C (metabotropic glutamate), Class D (fungal mating pheromone receptors), Class E (cyclic AMP receptors) and Class F (frizzled/smoothened). Out of these, only classes D and E are not found in vertebrates (Kolakowski 1994).

An alternative classification scheme called "GRAFS" divides vertebrate GPCRs into five classes (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, Secretin) (Schioth and Fredriksson 2005, Bjarnadottir, Gloriam et al. 2006).

Furthermore, the International Union of Basic and Clinical Pharmacology (IUPHAR) published a concise guide for the nomenclature of GPCRs which I will use in this thesis (Alexander, Benson et al. 2013).

The largest class by far is the rhodopsin-like receptor (Class A), which accounts for nearly 85 % of the GPCR genes. Frizzleds (FZDs) form a significantly smaller group, consisting of 10 FZDs and Smoothened (SMO) that are known to be seven-pass membrane proteins with receptor function. Despite the small size of this group, the complexity is demonstrated by the fact, that 10 FZDs can be activated by various ligands from the wingless/int (WNT) protein family consisting of 19 different members. Any combination between WNTs and FZDs, and thus their activation, is theoretically possible.

However, very little is known about the specificity and selectivity between different WNTs and FZDs (Schulte 2010).

WNT signaling pathways have been extensively studied and they are known to be crucial for cell-to-cell communication, differentiation, and morphogenesis during embryonic development and later in adulthood. Not surprisingly, the deregulation of WNT signaling is manifested in a high number of developmental defects, inherited diseases, and many types of cancer (Clevers 2006).

Even though FZDs consist of seven trans-membrane domains and according to the International Union of Basic and Clinical Pharmacology are classified as a group of GPCRs, it is still a matter of debate if FZDs indeed signal through heterotrimeric G proteins in a physiologically relevant manner.

This thesis aims to give a more detailed understanding of WNT receptors with a strong emphasis on FZD_6 by investigating the receptor within the cell membrane itself and combine these findings with the mechanism, which underlie binding of WNTs to FZDs and subsequent signal initiation.

1.1 THE WORLD OF G PROTEIN-COUPLED RECEPTORS

The very first explicit postulation for the existence of membrane-embedded receptors goes back to the British pharmacologist J. N. Langley. He wrote in 1905:

”I conclude then that in all cells two constituents at least must be distinguished, 1) substance concerned with carrying out the chief functions of the cells, such as contraction, secretion, the formation of special metabolic products, and 2) receptive substances especially liable to change and capable of setting the chief substance in action.” (Langley 1905)

Langley postulated two very important functions of these hypothetical receptor structures: 1) They react on chemical substances and stimuli probably due to specific binding and 2) they operate on effectors within the cell and can alter their function.

Since then time has passed and many researchers have questioned Langley. However, the work on GPCRs became so important and valuable that two Nobel Prizes were awarded so far in this research area. Alfred G. Gilman and Martin Rodbell were given in 1994 the Nobel Prize in medicine "for their discovery of G proteins and the role of these proteins in signal transduction in cells". Eighteen years later, in 2012, Robert Lefkowitz and Brian K. Kobilka were awarded the Nobel Prize in Chemistry "for their studies of G protein–coupled receptors".

But what is so special about these GPCRs?

GPCRs are composed of seven transmembrane helices. The binding sites for small and medium ligands are located in the membrane interior and for larger ligands rather in the extracellular regions of the receptor.

The N-terminus is extracellular and the C-terminal end is localized inside the cell. A disulfide bridge, which covalently connects the first and second intracellular loop is conserved in many GPCRs. Due to sequence homologies within the seven trans-membrane-domains (7TMD), GPCRs can be divided into different classes as mentioned previously (Kolakowski 1994, Schioth and Fredriksson 2005, Bjarnadottir, Gloriam et al. 2006, Alexander, Benson et al. 2013).

When a ligand binds to its receptor, the binding site to G proteins is exposed by a conformational transition. Once the association of an active ligand-receptor complex is achieved, it subsequently leads to a ligand-receptor-G protein complex and the initiation of a signal cascade (Kobilka 2007).

While the function of classical GPCRs is already fairly well understood, FZDs are one of the less studied family of GPCRs (Schulte 2010).

1.1.1 Discovery of the Frizzled Class of receptors

The so-called *frizzled* gene was first identified in *Drosophila melanogaster* in a screen for mutations that disrupt the polarity of epidermal cells in the adult fly (Wang, Macke et al. 1996). The discovered and responsible pathway – known as Planar Cell Polarity (PCP) - is key for the proper development of tissue polarity and has been identified in the 1980s (Vinson, Conover et al. 1989, Slusarski, Corces et al. 1997, Strutt, Weber et al. 1997, Gubb, Green et al. 1999). Key members of the PCP pathway are FZDs (Vinson, Conover et al. 1989), Dishevelled (Slusarski, Corces et al. 1997), Van Gogh (Vang) (Taylor, Abramova et al. 1998) and starry night (also known as Flamingo) (Chae, Kim et al. 1999).

FZDs can be found in a vast variety of organisms ranging from humans over *Drosophila melanogaster*, *Caenorhabditis elegans* to sponge *Suberites domuncula* (Adell, Nefkens et al. 2003) and *Hydra vulgaris* (Minobe, Fei et al. 2000).

Ten isoforms of FZDs can be found in vertebrates, four in *Drosophila melanogaster* and three in *Caenorhabditis elegans* (Huang and Klein 2004).

All vertebrate isoforms can be divided into four main clusters based on their homology (Fredriksson, Lagerstrom et al. 2003). FZD_1, FZD_2 and FZD_7 form the first cluster sharing approximately 75 % identity whereas the second group formed by FZD_5 and FZD_8 share 70 %. The third cluster comprised by FZD_4, FZD_9 and FZD_{10} share 65 % and the last cluster (FZD_3 and FZD_6) share 50 % amino acid identity. All FZD genes from different clusters share between 20 % and 40 % sequence similarity.

FZDs are bound and activated by the lipoglycoproteins of the WNT family (Willert, Brown et al. 2003; Schulte, 2010). However, expect of WNTs other molecules have been described to bind and activate FZDs. For example, Norrin – a protein that in humans is encoded by the *NDP* gene – was shown to bind and activate FZD_4 to subsequently induce β-catenin stabilization (Niehrs 2004, Xu, Wang et al. 2004). Dickkopf (DKK), Wise/Sclerostin and WNT inhibitory factor (WIFs) have been shown to be upregulated, when WNT signaling is activated, therefore antagonizing it (Niehrs 2006, MacDonald, Tamai et al. 2009, Cruciat and Niehrs 2013). Soluble Frizzled-related proteins (SFRPs) contain a FZD-like cysteine rich domain, which can bind and sequester WNTs (Hsieh, Rattner et al. 1999, Jones and Jomary 2002). However, SFRPs under certain circumstances, also bind and activate Frizzled receptors (Bovolenta, Esteve et al. 2008).

Furthermore, R-spondin is known to act through leucine-rich repeat-containing G protein-coupled receptors (LGRs) thereby sensitizing cells to WNT responses (Carmon, Gong et al. 2011).

Every receptor in the FZD family has a signal sequence in the very end of the extracellular N-terminal that is required for adequate insertion of the protein into the membrane. This signaling sequence is followed by a cysteine-rich domain (CRD), which is the orthosteric ligand binding site that binds WNTs (Dann, Hsieh et al. 2001, Janda, Waghray et al. 2012)

1.1.2 The WNT family

The first member of the WNT ligand family was *int1*, which was discovered in mouse, where it was activated as a proto-oncogene by the integration of the mouse mammary tumor virus (MMTV), leading to the development of breast cancer (Nusse and Varmus 1982). With the identification of Wingless (wg), the WNT1 ortholog in *Drosophila melanogaster* (Cabrera, Martinez-Arias et al. 1987, Rijsewijk, Schuermann et al. 1987), it became clear that WNT genes are also significantly involved in embryonic development (Nusse and Varmus 1992). Their mutations were shown to contribute to disturbances in the wings architecture as well as in the segment polarity (Sharma, Sharma et al. 1973, Nusslein-Volhard and Wieschaus 1980). The term "WNT" originated from the fusion of "Wingless" and "Integration 1" (Nusse and Varmus 1992).

WNT proteins not only play a key role in the regulation of various stages of embryonic development, such as the creation of patterning, but they are also involved in controlling the differentiation, proliferation, invasion, polarity and apoptosis of cells (Miller 2002). In adulthood, disturbances of the WNT signaling pathways can cause various diseases such as many types of cancer (Polakis 2000), osteoporosis (Patel and Karsenty 2002) and many others.

The human WNT family comprises of 19 different members that appear highly conserved in a variety of organisms.

WNTs are secreted as lipid modified glycoproteins, which have a length of 350 to 400 amino acids (aa). Other characteristic features of WNTs include a signal sequence for their secretion, a few highly charged residues and a large number of N-linked glycosylation sites (Willert, Brown et al. 2003). In addition, WNT proteins are characterized by a highly conserved distribution of 23 cysteines.

In addition to the classification of WNTs due to their amino acid sequence homology (Miller 2002), a further classification of the WNTs has been established considering the ability of individual WNTs to induce the transformation of murine mammary gland cells (C57MG) and thus cause breast cancer (Wong, Gavin et al. 1994, Polakis 2000). Members, which cause a strong transformation, were WNT-1, WNT-3, WNT-3A and WNT-7A. Little or no transformative causing WNTs were WNT-2, WNT-4, WNT-5A, WNT-5B, WNT-6, WNT-7B and WNT-11 (Kikuchi and Yamamoto 2007, Kikuchi, Yamamoto et al. 2007).

Historically, WNT-induced signaling has been classified into “canonical WNT signaling” and "non-canonical WNT signaling" based on the involvement of β–catenin. However, this classification is no longer retained since it was shown that canonical WNT-3A is also able to induce β-catenin independent signaling through the activation of Rho and Rho kinases (Kuhl, Sheldahl et al. 2000) or G proteins (Halleskog and Schulte 2013). Moreover, under certain conditions, WNT-5A (previously classified as “non-canonical WNT”) can induce both β-catenin dependent as well as β-catenin-independent pathways (Mikels and Nusse 2006).

1.1.3 WNT/FZD signaling

In general, there are at least three different branches of WNT signaling pathways described in the literature. The rather well-studied β-catenin-dependent WNT/β-catenin signaling pathway, as well as β-catenin-independent WNT/PCP (planar cell polarity) - and WNT/Ca^{2+} signaling pathway, which have been extensively studied in *Drosophila* and *Xenopus*. However, little is known about their biological function in mammals.

The WNT/PCP pathway is known to control cell polarity and cell movements during gastrulation (Heisenberg, Tada et al. 2000) and especially FZD_3 and FZD_6 seem to have a major role in neural tube closure and in the planar polarity of inner-ear sensory hair cells (Wang, Guo et al. 2006).

WNT/PCP-signaling leads to intracellular polarization of proteins such as Dishevelled (DVL), Prickle and Vangl, resulting in activation of small GTPases such as Rho and Rac, and consequently in oriented alignment of the cytoskeleton filaments.

The WNT/Ca^{2+} signaling pathway has been linked to an increase in intracellular Ca^{2+} concentration through heterotrimeric G protein activated phospholipase C and phosphodiesterase (Slusarski, Corces et al. 1997, Ahumada, Slusarski et al. 2002). The intracellular Ca^{2+} increase leads to a further activation of calcium/calmodulin-dependent protein kinase II (CaMKII), and the protein kinase C (Kuhl, Sheldahl et al. 2000). Although their importance is not fully understood yet, a function for cell proliferation and migration is suspected (Kuhl, Sheldahl et al. 2000).

Further function of the WNT/Ca2+ signaling pathway appears to be the inhibition of the WNT/β-catenin signaling pathway.

Here, the CamKII- and TAK1-mediated activation of mitogen-activated protein kinase NLK (NLK-MAPK), phosphorylates the T-cell factor (TCF), and thereby inhibits the binding of the β-catenin/TCF complex to the DNA and thus prevents the transcription (Ishitani, Kishida et al. 2003).

In the context of heterotrimeric G proteins, it was reported that WNT-5A induces G protein-dependent signaling to ERK1/2 and WNT-5A was linked to the regulation of proinflammatory responses in mouse primary microglia cells (Halleskog, Dijksterhuis et al. 2012).

1.1.4 Frizzled 6

Historically, FZD_6 has been linked to the PCP pathway, where it is important for the proper orientation of hair follicles (Wang, Badea et al. 2006), auditory sensory cells and for neural tube closure (Wang, Guo et al. 2006). Furthermore, it has been shown that FZD_6 negatively regulates the β-catenin dependent pathway (Golan, Yaniv et al. 2004). Despite a positive correlation between FZD_6 expression and WNT-3A induced β-catenin activation in human mesenchymal stem cells was shown, the knockdown of FZD_6 in these cells had no influence on this activation (Kolben, Perobner et al. 2012).

Lately it has been shown that mutation of FZD_6 can result in defects in nail and claw formation. The mutated form of FZD_6 shows abolished membranous levels and nonfunctional WNT/FZD signaling in mammals (Frojmark, Schuster et al. 2011). In this context it is not surprising that FZD_6 deficiency disrupts the differentiation process of nail development (Cui, Klar et al. 2013). Furthermore, FZD_6 is a critical component linked to the development of B cell lymphocytic leukemia (Wu, Zierold et al. 2009).

For the purpose of this thesis it is important to mention that FZD_6 is highly expressed in the lung epithelium and leads to cancer once miss-regulated occurs (van Helden, Godschalk et al. 2010, Piga, van Dartel et al. 2014)[1].

Using a mono color FRAP approach, our group has previously analyzed the WNT-isoform-specific abilities to promote changes in FZD_6 molecular mobility and grouped them accordingly. Among the WNTs tested, fast and transient mobility shifters were: WNT-2B and WNT-7A. Intermediate and transient shifters were: WNT-1, WNT-5A, WNT-9B and WNT-10B and slow and persistent shifters were WNT-3A and WNT-4. WNT-5B and WNT-11 did not show any affect in the molecular mobility upon stimulation (Kilander, Dahlstrom et al. 2014).

Most interestingly, with the use of pertussis toxin and pertussis toxin-resistant mutants of $G\alpha_i$, the study linked the change in mobility shift to the dissociation of the heterotrimeric G protein (in case of WNT-5A) (Kilander, Dahlstrom et al. 2014).

1.1.5 Other WNT receptors: LRP5/6, ROR1/2, Vangl1/2 and RYK

In addition to FZDs, other membrane receptors have been associated with WNT signaling, which I will describe only briefly:

1.1.5.1 LRP5/6:

The low-density lipoprotein-related proteins 5 and 6 (LRP5 and LRP6) belong to the type I transmembrane proteins with a single membrane spanning domain and play an essential role in the signal transduction of WNT/β-catenin signaling (Pinson, Brennan et al. 2000).

[1] http://www.proteinatlas.org/ENSG00000164930-FZD6/cancer/tissue/lung+cancer

The extracellular N-terminus of LRP5/6 represents the main part of the protein (He, Semenov et al. 2004), and an interaction of this domain with various WNT proteins was experimentally be detected. WNT-1 and WNT-4 were shown to bind LRP5 (Mao, Wang et al. 2001, Kato, Patel et al. 2002), whereas WNT-1, WNT-3A, WNT-9B and XWNT-8 show preferential binding to LRP6 (Itasaki, Jones et al. 2003, Liu, Bafico et al. 2003, Tamai, Zeng et al. 2004, Bourhis, Tam et al. 2010). However, the WNT/LRP5/6 interaction seems to be significantly weaker compared to WNT/FZD binding (He, Semenov et al. 2004). Recent evidence also indicates that selective recruitment of LRP5/6 and receptor tyrosine kinases such as ROR1/2 and RYK, is important for WNT signaling outcome (Hendrickx and Leyns 2008, van Amerongen, Mikels et al. 2008).

However, it appears that LRP5/6 cannot initiate WNT/β-catenin signaling without the presence of FZDs (Dijksterhuis, Baljinnyam et al. 2015) and DVL is required for the phosphorylation of LRP6 (Bilic, Huang et al. 2007)

1.1.5.2 ROR1/2:

Receptor tyrosine kinase-like orphan receptor 1 and 2 (ROR 1 and ROR2) play an important role in neuronal development, bone formation, and in the development of organs such as the lungs and brain (Minami, Oishi et al. 2010).

ROR1/2 have a similar CRD domain compared to FZD (Xu and Nusse 1998) and an intracellular kinase domain, which appears to be dispensable for the transfer to intracellular signaling cascades (Hikasa, Shibata et al. 2002).

In case of ROR1, it is thought that WNT-5A binds to the CRD of ROR1 and then interacts with FZD and initiates the β-catenin-independent signaling pathway. “Canonical” WNTs, on the other hand, were reported to antagonize ROR1 signaling (Grumolato, Liu et al. 2010).

ROR1 seems to have a high potential as a diagnostic and possibly as prognostic marker, since it is uniformly expressed in CLL patients when compared to healthy individuals (Baskar, Kwong et al. 2008, Uhrmacher, Schmidt et al. 2011)
Activation of the non-canonical JNK signaling pathway via ROR2 has been shown to be WNT-5A-dependent (Unterseher, Hefele et al. 2004). Further, overexpression experiments with ROR2 revealed an inhibitory function of this receptor on the β-catenin dependent WNT pathway (Mikels and Nusse 2006).

1.1.5.3 RYK:

The receptor-like tyrosine kinase (RYK) has an extracellular N-terminal domain, known to bind WNT proteins (Patthy 2000), and an intracellular PDZ domain. In experiments with human cells, interaction with WNT-1 and WNT-3A was shown to be followed by the activation of the β-catenin dependent WNT pathway. Furthermore, it has been suggested that RYK thereby forms together with FZD and WNT a trimeric complex that activates signal transduction via DVL (Lu, Yamamoto et al. 2004).

1.1.5.4 Vangl1/2:

Van Gogh-like protein 1 and 2 (Vangl1/2) are four-pass transmembrane proteins that are known to be components of the PCP pathway (Wu and Mlodzik 2009). Vangl2 and ROR2 are known to act together to transduce the signal after the binding of WNT-5A. ROR2 forms a receptor complex with Vangl2 and phosphorylates Vangl2 in a WNT-dependent manner, which is subsequently essential for establishing the planar cell polarity via Rho and JNK (Gao, Song et al. 2011).

1.1.6 FZD binding partners

FZD receptors are known to interact directly or indirectly with many proteins involved in WNT signaling such as LRP5/6, DVL1/2/3 and various G proteins.

So far, it is not well described how exactly DVL interacts with FZD. Is full activation of the receptor leading to the binding of DVL to the receptor or does DVL already exist in a pre-associated state? In other words, it is still unknown whether the interaction between DVL and FZDs is static or dynamic and how agonist treatment is altering this interaction.

This is rather difficult to analyze and study since available DVL antibodies are poor for detection of endogenous DVL and co-expression with FZDs recruits DVL to the membrane (Cong, Schweizer et al. 2004).

The DVL protein consists of three main domains: the N terminal Dishevelled and Axin (DIX) domain, the Dishevelled, Eg-10 and Pleckstrin (DEP) domain and the Psd-95/Disc large/ZO-1 homologous (PDZ) domain. The DIX domain is the main domain involved in polymerization of DVL and the proteins typical punctate appearance when overexpressed in vitro (Schwarz-Romond, Merrifield et al. 2005). The PDZ domain enables DVL to interact with other proteins including Class Frizzled receptors whereas the DEP domain is functionally involved in the regulation of small GTPases. DVL has been shown to be involved in the β-catenin-dependent as well as the –independent pathways such as PCP, WNT/RAC, WNT/RHO and possibly also the WNT/Ca^{2+} pathway (Gao and Chen 2010).

Activation of the WNT/PCP signaling pathway requires the translocation of DVL to the plasma membrane pointing at the importance of the cellular localization of DVL (Park, Gray et al. 2005).

FZDs are - despite all debates - thought to interact with heterotrimeric G proteins. In silico analysis proposed interaction of FZDs with G proteins specifically of the $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_s$ groups (Moller, Vilo et al. 2001). In vivo studies showed pertussis toxin sensitive inhibition of the FZD_1 mediated β-catenin-dependent WNT signaling pathway through $G\alpha_o$. Additionally, WNT/β-catenin signaling could effectively be blocked by the knockdown of $G\alpha_q$ proteins (Liu, DeCostanzo et al. 2001). Experiments using loss-of-function mutations in DVL, overexpression of GSK3β and the use of mutant $G\alpha_o$ proteins in *Drosophila* further supported the function of FZDs as guanine nucleotide exchange factors (GEF) and localized G protein activity upstream of DVL (Katanaev, Ponzielli et al. 2005).

Inhibition of the FZD_2-mediated β-catenin-independent signaling pathway was also achieved by pertussis toxin treatment and knockdown experiments of $G\alpha_o$ and $G\alpha_{t2}$ (Liu, Liu et al. 1999, Ahumada, Slusarski et al. 2002), which supports the possibility of different G proteins being recruited to the same FZD depending on the activating signal.

Loss-of-function approaches indicated a functional role of heterotrimeric G proteins in WNT/FZD signaling (Slusarski, Corces et al. 1997, Liu, Liu et al. 1999, Liu, Liu et al. 1999, Sheldahl, Park et al. 1999, Katanaev, Ponzielli et al. 2005, Koval, Purvanov et al. 2011). More recently, experiments in mammalian cells and tissue preparations confirmed that WNTs can evoke GDP/GTP exchange at heterotrimeric G proteins at physiological stoichiometry of the involved signaling components (Katanaev and Buestorf 2009, Kilander, Dijksterhuis et al. 2011, Kilander, Halleskog et al. 2011, Koval, Purvanov et al. 2011, Halleskog and Schulte 2013).

Thus, even though WNT-FZD-G protein signaling is relevant in physiological processes, underlying details of FZD-G protein communication and its involvement WNT signaling remain unclear.

With regard to the FZD co-receptor LRP6 or the similar LRP5, it is generally believed that binding of WNT to FZDs leads to the formation of a WNT-FZD-LRP complex (van Amerongen, Mikels et al. 2008). This complex formation and the subsequent recruitment of the scaffolding protein DVL lead to the activation of LRP6/5 by CK1-mediated phosphorylation. Subsequently, the activated co-receptor causes a redistribution of Axin to the cell membrane and leaves a dysfunctional destruction complex in the cytosol (Stamos and Weis 2013). However, the underlying stoichiometry remains unclear (Schulte 2015). This causes β-catenin to accumulate in the cell and to enter the nucleus, where it binds to the transcription factors TCF/LEF and regulates the expression of a variety of proteins, including cyclin D1, c-myc, COX-2 and iNOS (Ramsay, Ciznadija et al. 2003, Barker 2008, Du, Zhang et al. 2009, MacDonald, Tamai et al. 2009). It has been thus proposed that WNTs induce co-clustering of FZDs and DVL in LRP6-signalosomes, which in turn triggers the phosphorylation of LRP6 and promote Axin recruitment and β-catenin stabilization (Bilic, Huang et al. 2007)

1.1.7 Structure, function and transport

Upon activation by extracellular agonists, GPCRs transmit signals to the cell interior. Ligands can bind GPCRs either on the extracellular N-terminus and part of the extracellular loops (for example glutamate receptors) (Niswender and Conn 2010) or to a well-defined binding pocket within the transmembrane helical bundle (Rhodopsin) (Landau, Pebay-Peyroula et al. 2003).

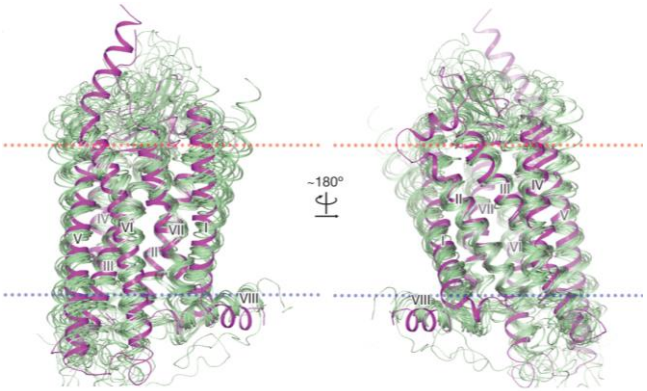


Figure 1: Superimposing the inactive conformational structures of the SMO receptor (magenta) and class A GPCRs (light green) from the side (Wang, Wu et al. 2013)

All GPCRs are activated either by agonists, or through a spontaneous auto-activation of receptors, referred to as constitutive activity (Trzaskowski, Latek et al. 2012). Full activation of GPCRs is acquired through a conformational change "switching" the receptor from its inactive to its active state. This type of "switching" has been previously described for classical GPCRs. The amino acids which form the so called D(E)RY motif in most of the Class A GPCRs are conserved Glu, Arg and Tyr on the cytoplasmic end of transmembrane domain 3 (TM3) (Fahmy and Sakmar 1993).

The Arginine is, with 96 % homology, the most conserved amino acid in Class A GPCRs (Mirzadegan, Benko et al. 2003). In the inactive conformation of the receptor, a number of hydrogen bonds are formed with the neighboring amino acids Glu, Glu and Thr (Ramon, Cordomi et al. 2007, Cordomi, Ramon et al. 2008), through which TM3 connects with TM6 and the charge of the Arg within the D(E)RY motif is shielded/neutralized. This network surrounded by the arginine is referred to as "ionic lock" (Vogel, Mahalingam et al. 2008). Upon stabilization of the active conformation, a proton is added to this network (Arnis, Fahmy et al. 1994, Knierim, Hofmann et al. 2007), de-stabilizing the charge between the helices whereupon the side chain of the arginine is used as docking site for the G protein.

Opening of the lock is accompanied by large conformational changes of TM6 in the agonist-G protein-bound β_2 adrenergic receptor complex (Rasmussen, DeVree et al. 2011). However, even though the D(E)RY motif exists in almost all class GPCRs (Rosenbaum, Rasmussen et al. 2009), an "ionic lock" has not been identified in all GPCRs. The "ionic lock" in the β_1-adrenergic receptor, for example, does not seem to have an essential role for the maintenance of the inactive state (Warne, Serrano-Vega et al. 2008).

With regard to Class Frizzled receptors, which do not contain a D(E)RY motif, a mechanism corresponding to the "ionic lock" has so far not been identified. In addition, there is no crystal structure for any FZD reported so far, which makes it difficult to pinpoint certain amino acids that could be relevant for FZD activation. However, the high resolution crystal structure of the closely-related smoothened (SMO) receptor has previously been published (Wang, Wu et al. 2013).

Interestingly, when superimposing the structures of SMO and class A GPCRs, all receptors - despite their homology and functional differences – appear to be structurally quite similar (**Figure 1**). However, most of the conserved motifs for class A GPCRs are lacking and the structure reveals an unusually complex arrangement of long extracellular loops (Wang, Wu et al. 2013).

With respect to FZDs, the G protein–receptor interface within the third intracellular loop (i3) and the C-terminus (Wess 1998) overlaps substantially with regions important for FZD–DVL interaction (Wong, Bourdelas et al. 2003, Tauriello, Jordens et al. 2012). This overlap raises the question whether steric hindrance or close collaboration play a role for FZD–DVL and FZD–G protein interaction, signal integration and signal specification.

1.1.8 Oligomerization

For a long time, it was believed that GPCRs exist exclusively as monomers. However, in the late 90s, first oligomerization on the vasopressin V2 receptor (V2R) (Schoneberg, Yun et al. 1996), the gonadotropin-releasing hormone receptor (GnRHR) (Grosse, Schoneberg et al. 1997) and the δ- opioid receptor (Cvejic and Devi 1997) was shown. Since then, a vast number of GPCRs were shown to form oligomeric complexes. Even in tissue, where GPCRs are expressed endogenously, oligomerization of receptors has been detected (Albizu, Cottet et al. 2010). Nevertheless, how GPCR oligomerization takes place at the molecular level and what is the role of the oligomers in receptor function and pharmacology has only been resolved for a few GPCRs. For the prototype GPCRs, rhodopsin, β2-adrenergic- and the μ-opioid receptor, it was shown that a monomer is sufficient for the binding of a G protein (Ernst, Gramse et al. 2007, Whorton, Bokoch et al. 2007, Whorton, Jastrzebska et al. 2008, Kuszak, Pitchiaya et al. 2009, Rasmussen, DeVree et al. 2011). In the case of rhodopsin, a monomer was detected as the smallest functional unit sufficient for the binding of β-arrestin and for the phosphorylation through GRK1 (Scheerer, Park et al. 2008, Tsukamoto, Sinha et al. 2010, Bayburt, Vishnivetskiy et al. 2011). Nevertheless, it is widely assumed that the reason for an interaction between GPCRs is not always the activation of signal transduction itself. Rather cooperative and allosteric effects have been described (Nature Reviews Drug Discovery 2004).

From a pharmacological point of view it is of particular interest to study the possibility of different signaling pathway activation via the formation of GPCR oligomers.

As an example, reduction of $G\alpha_i$-coupling by co-expression of δ- and μ-opioid receptors (George, Fan et al. 2000, Charles, Mostovskaya et al. 2003) or CCR5 and CCR2 chemokine receptors (Mellado, Rodriguez-Frade et al. 2001) has been reported. With respect to homo-oligomers of the TSH-Receptor, however, both $G\alpha_s$ and $G\alpha_q$ are activated: The TSH hormone binds first to the N-terminal high-affinity binding site on the receptor followed by $G\alpha_s$ C-terminal coupling. When the low-affinity site on the N-terminus is occupied by TSH, an additional $G\alpha_q$ coupling to the oligomer occurs (Allen, Neumann et al. 2011). Another mechanism lies in the initiation of signal transduction via transactivation within a GPCR dimer. Here, the ligand binds initially at the N-terminus of one receptor. This ligand then activates the G-protein coupling to the neighboring receptor. This type of activation has been shown by signal deficient mutants of the luteinizing hormone receptor that are only in combination capable of initiating a signaling cascade (Rivero-Muller, Chou et al. 2010).

There are also GPCR oligomers described, which play an important role in their transport to the plasma membrane (PM) or in their internalization.

The best known example here is the $GABA_B$ receptor that can be transported from the endoplasmic reticulum (ER) to the plasma membrane only as an oligomer. The subunit $GABA_{B1}$ cannot access the plasma membrane due to a coat protein-complex I (COPI) binding site on the C-terminus. COPI is a protein complex that forms the shell of retrograde transport vesicles in the secretory pathway. Only after interaction with $GABA_{B2}$ this binding site is hidden. While $GABA_{B2}$ is also transported without the presence of $GABA_{B1}$ to plasma membrane, the converse is not possible. However, both subunits of the GABA receptor are important for signaling: $GABA_{B1}$ for ligand binding and $GABA_{B2}$ for the G protein coupling (Pin, Comps-Agrar et al. 2009).

In the mentioned phenomena it is, in most cases, unclear whether the interacting GPCR exist only as dimers or even higher oligomers and whether these di- or oligomers exist in a fixed, possibly functional, stoichiometry of monomers and dimers (or oligomers).

Ligand-induced rearrangement, but not dissociation, of a dimeric metabotropic glutamate receptor (mGluR) was linked to receptor activation (Xue, Rovira et al. 2015), whereas for the M1 muscarinic, β1-adrenergic and $GABA_B$ receptor, dimer formation and dissociation of a dimer could be observed by single molecule tracking (Hern, Baig et al. 2010, Calebiro, Rieken et al. 2013). The dynamics of oligomers, however, is overall poorly studied.

In the case of receptor tyrosine kinases an influence of the monomer-dimer relationship (M/D) is known for its activity (Maruyama 2014). However, such monomer-dimer relation in regard to GPCRs regulation has hardly been taken into consideration.

1.1.9 Mechanisms of G protein activation

The activation of G proteins requires an activated GPCR. In order to clarify the interplay between G protein and GPCR or in other words - how G proteins reach an activated receptor - I would like to introduce two different models which are being discussed in the field (Hein and Bunemann 2009). Receptor and G protein can interact by catalytic collision interaction (collision coupling) (Hein, Frank et al. 2005, Digby, Lober et al. 2006), which means that a single receptor can possibly activate multiple G proteins in succession. A collision coupling mechanism for the β2-adrenergic receptor (B2AR) has been demonstrated (Hein, Frank et al. 2005) and it has been estimated that the β-adrenergic receptor is able to activate about 100 $G\alpha_s$ molecules in native membranes (Ransnas and Insel 1988).

Furthermore, the α2-adrenergic receptor for example interacts with $G\alpha_i$ via a collision mechanism (Hein, Frank et al. 2005).

This mechanism explains in a more plausible way the observed signal gain on G protein level, where a receptor can activate more G proteins at the same time. The accumulation of G proteins to particular cell compartments or specializations of the plasma membrane furthermore favors their selective interactions with the receptor (Huang, Hepler et al. 1997).

As an alternative model, varying degree of pre-coupling, pre-assembly or inactive-state preassembly between receptor and G protein are discussed (Bruheim, Krobert et al. 2003, Qin, Dong et al. 2011, Drastichova and Novotny 2012). The pre-coupling, pre-assembly or inactive-state preassembly models can in part explain the specificity of a GPCR-G protein complex and the rapid intracellular signal response. However, the effect on signal amplification has its limitations when considering these types of models. Here, the so called collision coupling model that ensures rapid exchange of G proteins, seems to be necessary to provide the enormous amplification reported for example for rhodopsin (Oldham and Hamm 2008).

Nevertheless, one cannot rule out the possibility that a receptor changes from one model to the other and therefore ensures both: rapid signaling and signal amplification. Signal amplification can also occur downstream of G protein activation. Localization of proteins in membrane signaling microdomains, such as caveolae and lipid rafts, can further promote one model over the other.

In the absence of a ligand, the G alpha subunit is GDP bound and in complex with Gβγ. The activation of a receptor results in a high affinity binding of the G alpha subunit to the receptor.

The receptor then acts as guanine nucleotide exchange factor (GEF), thereby supporting the release of GDP. This is followed by the binding of GTP, which is present in significantly higher concentration than GDP in cells. The guanine nucleotide exchange results in a conformational change of the G alpha subunit, which either leads to the dissociation of G alpha and the Gβγ subunits or causes a rearrangement of these subunits (Hepler and Gilman 1992, Bunemann, Frank et al. 2003). Thereupon, Gα and also the Gβγ subunits activate downstream events (Ford, Skiba et al. 1998). The Gα subunit contains an intrinsic GTPase activity that hydrolyzes GTP to GDP and thus leads to the re-association of the Gα subunit with the Gβγ complex, which terminates the cycle. However, experiments with purified G proteins suggested that the GTPase activity is biochemically considered too slow to terminate physiological processes (in the range of 10-20 seconds for most Gα proteins 50 seconds for $G\alpha_q$ and up to 7 min for $G\alpha_z$ (Gilman 1987, Berstein, Blank et al. 1992). This contradiction led to the postulation of regulatory proteins (regulators of G protein signaling [RGS]) accelerating the GTPase activity, which could be isolated and their GTPase-activating function has been demonstrated (Ross and Wilkie 2000).

1.1.10 Ligands and G proteins

Specific binding of a ligand to its receptor is a prerequisite for most of the drug effects. GPCRs can trigger a number of different signaling cascades within the cell after the specific binding of a particular ligand. According to their effects on signal transduction, ligands may be present as 1) **full agonists** 2) **partial agonists** 3) **neutral antagonists** 4) **inverse agonists** (Rosenbaum, Rasmussen et al. 2009). In addition, in recent years, biased agonism or ligand bias for GPCRs, has been introduced.

It describes the functional selectivity of a ligand (having many different efficacies), activating upon binding to the receptor one or several possible signal pathways, also referred to as "pluridimensional efficacy" (Violin and Lefkowitz 2007, Kenakin 2011, Whalen, Rajagopal et al. 2011, Reiter, Ahn et al. 2012). More recent studies show that GPCRs can bypass G proteins and initiate G protein-independent signaling through β-arrestin. Thus, they are able to act as agonists or inverse agonists at the same receptor and activate different signaling pathways (Galandrin, Oligny-Longpre et al. 2007). In the same year Violin and Lefkowitz confirmed that "biased-ligands" can selectively activate G proteins or β-arrestin (Violin and Lefkowitz 2007). For the chemical compound JNJ7777120, for example, it was shown that it was a "biased ligand" for the Histamine H4 receptor that selectively activates β-arrestin, independent of the G Protein (Rosethorne and Charlton 2011).

In the literature, many terms for biased agonism have been used: "agonist-directed trafficking of receptor stimulus", "Agonist trafficking of receptor signals" (Kenakin 1995, Berg, Maayani et al. 1998), "functional selectivity", "biased agonism" (Kenakin 2007), "ligand biased efficacy", "collateral efficacy", "pluridimensional efficacy" (Galandrin and Bouvier 2006).

Such "biased signaling" offers many new opportunities to target and modulate specific signaling pathways and thus intervene with pathophysiological processes (Violin and Lefkowitz 2007, Kenakin 2011).

Biased signaling in terms of WNT/FZD signaling has been proposed in a systematic mapping study, where distinct WNT-FZD interactions where related to individual signaling (Kilander, Halleskog et al. 2011, Dijksterhuis, Baljinnyam et al. 2015). Furthermore, there is evidence that a single FZD can activate multiple signaling pathways.

FZD_3, FZD_4 and FZD_5 for example can act through possibly both, the β-catenin dependent and independent WNT signaling pathway (Burns, Zhang et al. 2008). This is probably depending on the interaction of FZDs with downstream proteins or on the combination of FZDs binding to different WNTs (Mikels and Nusse 2006, van Amerongen, Mikels et al. 2008).

However, the underlying mechanisms that determine the outcome of WNT-induced FZD activation are still poorly understood. First of all, the degree of WNT-FZD binding specificity is largely unknown and the question if certain WNT-FZD combinations could selectively activate certain signaling routes over others has not been systematically addressed. Secondly, coupling selectivity of activated FZDs to downstream signaling pathways remains obscure.

It has been shown that receptors can couple to more than one G protein isoform (Kenakin 1995).

This is exemplified by the cannabinoid CB1 receptor: The ligand "desacetyllevonantradol" acts with respect to $G\alpha_{i1}$ and $G\alpha_{i2}$ as a positive agonist, however, in regard to $G\alpha_{i3}$ as an inverse agonist. "Methanandamide" is an inverse agonist for $G\alpha_{i1}$ and $G\alpha_{i2}$, and a positive agonist for $G\alpha_{i3}$ (Mukhopadhyay and Howlett 2005). Corresponding observations could be justified by different degrees of ligand selective conformations at the receptor. This hypothesis, however, would be incompatible with the classic concept of the "two state model".

2 SPECIFIC AIMS

This thesis aims to uncover new structural and functional aspects of WNT receptors with a strong emphasis on FZD_6 and to provide a new perspective on molecular features of FZD in regard to the future use for drug development. The specific aims were in particular:

- Solving the longstanding question of FZD-G protein coupling.
- Obtain a more detailed and structural-function understanding of Class Frizzled receptors.
- Assess FZD_6 dimerization and the functional relevance of agonist induced dynamics
- Role of DVL for FZD_6-G protein coupling
- Provide new tools to study FZDs for future research on Class Frizzled receptors
- Develop a mammalian readout assay for the analysis of PCP protein activity and their asymmetric localization

3 MATERIALS AND METHODS

3.1 FLUORESCENT RECOVERY AFTER PHOTOBLEACHING

Fluorescent recovery after photobleaching (FRAP) is a very powerful tool to investigate the dynamics of proteins within living cells. The method describes the measurement of the recovery of fluorescence in a defined area in a microscopic image after previous bleaching of the fluorescence in this particular area (**Figure 2**). With this method the mobility and diffusion rate of the analyzed proteins can be determined and analyzed. For this purpose, a fluorescent area is selected, the fluorescence intensity measured and then bleached by a short but strong laser pulse only applied at this location. Photobleaching is irreversibly destructing the fluorescent molecules, which is causing it to become non-fluorescent. Subsequently, the intensity in the selected area is being followed for a certain period of time, usually seconds to minutes, thereby detecting the time it takes for fluorescent proteins to migrate into this area.

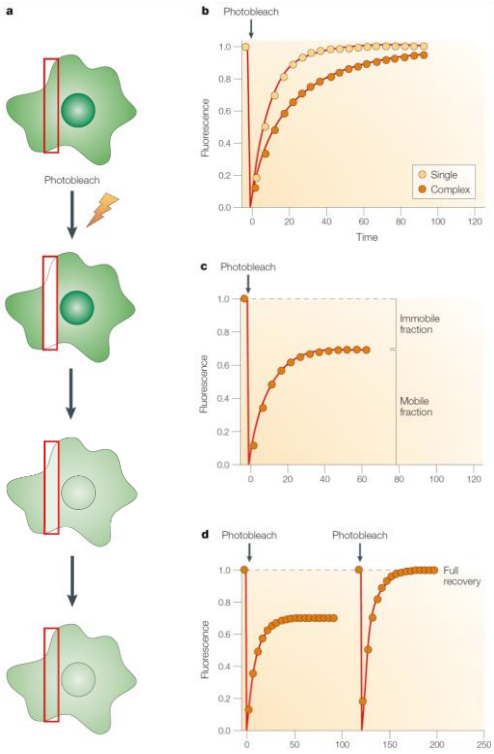


Figure 2 Fluorescence intensities of a Bleached-ROI in a time series, the bleached area is marked red. Illustrated are different types of possible kinetics depended on the properties of the proteins. For all examples a region of interest (ROI) within a cell is bleached (red box). Non bleached molecules are moving into the ROI. The speed of redistribution can be determined (Lippincott-Schwartz, Altan-Bonnet et al. 2003)

The gained intensity of the recovery curve is then needed to calculate the diffusion coefficient, from which the percentage of the mobile proteins can be determined (**Figure 2**).

The modified dual color FRAP (dcFRAP) method is using two fluorescently labeled proteins allowing to study their interaction dynamics. Therefore cell surface or specific protein mediated cross-linking is needed (Dorsch, Klotz et al. 2009, Lambert 2009).

This method has been one of the main breakthroughs in **Paper I**, where we measured G protein binding to a FZD_6 receptor and monitored its dissociation upon agonist stimulation. In addition, with the modified dcFRAP method we were able to look for receptor-receptor interaction and its dynamics in **Paper II**.

3.2 FLUORESCENT CROSS CORRELATION SPECTROSCOPY

To overcome the issue of overexpressing certain proteins and artificially cross linking the cellular membrane of living cells another method has been used. Fluorescent cross-correlation spectroscopy (FCCS) is a very sensitive method with which near physiological relevant levels of receptors and proteins can be studied in living cells.

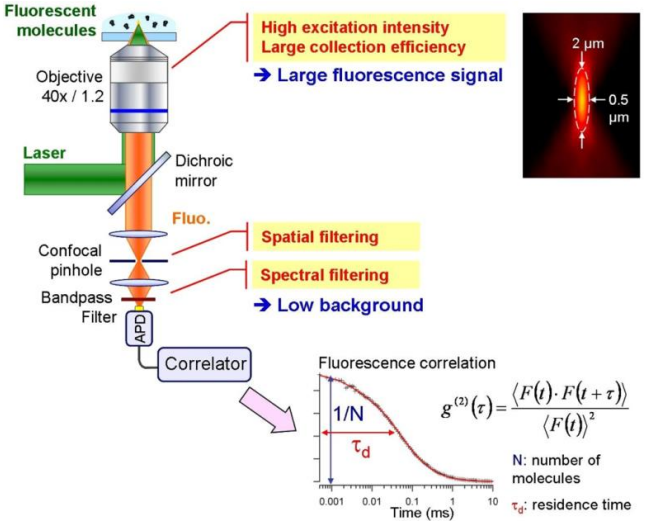


Figure 3 Principle of fluorescence correlation spectroscopy: the fluorescence intensity originates from a well-defined volume and are recorded and correlated to yield estimates for the average number of molecules observed and the characteristic fluctuation time (Wenger and Rigneault 2010)

Fluorescence correlation spectroscopy (FCS) was developed in the early 70s by Elson and Webb to measure the transport properties and concentrations of fluorophores in solution (Bopp and Magdeburg 1972, Webb 1976) . In FCS, a focused laser beam illuminates a voxel in a size of a femtoliter or smaller.

The fluorescent particles diffuse through the focus due to Brownian motion. The light, which is emitted by the fluorophores while they passively diffuse through the voxel is collected and detected using a confocal microscope.

Since the measurements take place in a very small observation volume, FCS is also considered as a single-molecule technology. When a volume of one femtoliter is observed with a 1 nM solution less than one particle (0.6) is observed within the focus. An important extension of FCS is FCCS, also known as two-color FCS (Schwille, Meyer-Almes et al. 1997). The principle based on FCCS is the simultaneous excitation and detection of two spectrally separated fluorophores. In addition to auto-correlation for each channel, the intensity fluctuations of both channels can be cross-correlated. Particles, that give a signal simultaneously in both channels, meaning both colors simultaneously diffuse through the volume when they interact with each other, contribute to the cross-correlation (**Figure 3**).

The cross-correlation amplitude provides information about the fraction containing two colors diffusing at the same time through the focus. A series of such in vitro binding studies have been conducted using the FCCS (Tewes, Tsikas et al. 1998) [2].

[2] Weidemann, T., Wachsmuth, M., Tewes, M., Rippe, K. and Langowski, J. (2002), Analysis of Ligand Binding by Two-Colour Fluorescence Cross-Correlation Spectroscopy. Single Mol., 3: 49–61. doi: 10.1002/1438-5171(200204)3:1<49::AID-SIMO49>3.0.CO;2-T

The FCCS is an elegant, biophysical method that slowly finds its application also in biological and pharmacological questions. Schmidt et al. showed with the help of FCCS that the oligomerization of the amyloid precursor protein (APP) is inhibited in the presence of the transmembrane Sortilin-related receptor (SORLA) (Schmidt, Baum et al. 2012).

Due to its single molecule sensitivity, this method allowed us in **paper II** to further dissect the dynamics in FZD_6 dimerization without the use of artificial cross linking. Together with dcFRAP, FCCS has been a very powerful tool in our experimental setup to link and analyze receptor-receptor and receptor-G protein dynamics.

3.3 CIRCULAR DICHROISM (CD)

The CD spectroscopy is an excellent method to gain helpful indications for secondary structure of a protein or peptide. The term circular dichroism (CD) refers to the different absorption of right and left circularly polarized light through a sample in the Ultraviolet-Visible Spectrophotometry (UV-Vis). In general, protein CD spectra can be divided into two characteristic spectral regions: i) absorbing in a range from 160 to 230 nm, the so called peptide region, and ii) a range of 230 to 300 nm, in which mostly aromatic acid are detected and absorbed. The CD spectra of proteins in the peptide range below 250 nm are sensitive for the detection of secondary structures. This applies in particular for the characterization of α-helical units (Sreerama and Woody 2004).

The UV-Vis CD spectrum of α-helix is characterized by a positive band at 192 nm and by two negative minima at 208 and 222 nm. Expectedly, coiled-coils show in CD experiments also the typical signals for α-helices.

While the absorption bands at 222 nm gives indications on the α-helical content, the 208 nm absorption band is an indication that the helix exists as single or is present in multiform, as in coiled-coils (Lau, Taneja et al. 1984).

In contrast to the CD spectrum of a helical structures antiparallel β-sheets are indicated by a positive absorption band at 195 nm and a negative signal at 218 nm (**Figure 4**). Typical CD spectra are detected in a range of 260-178 nm within 30 to 60 minutes.

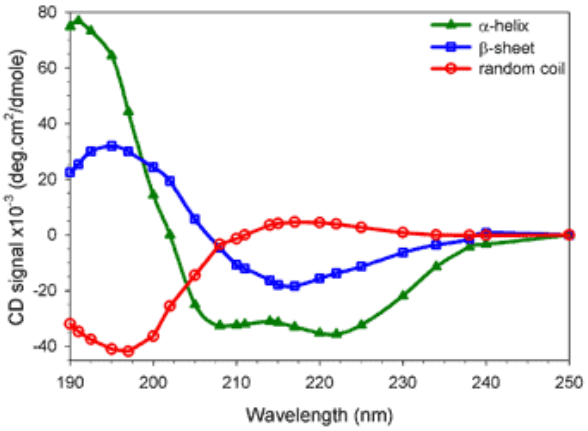


Figure 4 Characteristic UV-Vis circular dichroism (CD) spectra of α-Helix, a β-sheet and a disordered random coil structure[3].

Circular dichroism in combination with isothermal titration calorimetry (see below) was used in **paper II** to validate TM minigenes as tools to interfere with receptor dynamics.

3.4 THE ISOTHERMAL TITRATION CALORIMETRY

Isothermal titration calorimetry (ITC) is used to detect interactions between substances of equal or different molecule classes.

[3] http://www.fbs.leeds.ac.uk/facilities/cd/images/1.png

These range from interactions between two peptides, to hormone-receptor binding, up to interactions between antibodies and their cell surface proteins.
ITC measurements are performed by titrations. If two binding partners interact, heat is produced or released from the measuring system. This depends on whether it is an endothermic or exothermic reaction, respectively. This heat change is measured and used to calculate the change in enthalpy (ΔH). This value describes the change in the energy state of a system. In addition, by using titration, the calorimeter can be used to determine the affinity, or the Kd-value of the interacting partners (Freyer and Lewis 2008).

3.5 CELLULAR THERMAL SHIFT ASSAY

The folding of a protein is often stabilized by binding of other molecules. The stabilization is manifested among others by increased thermal stability of the protein in which the biological activity of the protein is retained even at relatively high temperatures and denaturion starts only at higher temperatures (Koshland 1958). The novel cellular thermal shift assay (CETSA) is very simple and requires no labeling with dyes compared to the previous known thermal shift assay (Niesen, Berglund et al. 2007). The CETSA method is most commonly used to measure the thermal stability for a given protein at different temperatures, with and without the presence of an active ingredient. The heating in this case takes just about a minute. When an active ingredient binds, the thermal stability of the protein is changing. This is referred to the melting curve (Martinez Molina, Jafari et al. 2013, Jafari, Almqvist et al. 2014). We have used this method in **Paper III** to compare the thermal shift of FZD_6-wt receptors compared to the mutant form thought to be influencing the conformation and therefore the thermal stability of the protein.

4 RESULTS AND DISCUSSION

4.1 FZD_6-G PROTEIN INTERACTION: COLLISION COUPLING VS INACTIVE STATE ASSEMBLY AND G PROTEIN SELECTIVITY

Functional interaction of ligand, GPCR and the heterotrimeric G proteins as transducers was summarized in the so called ternary complex model, which describes the receptor-dependent allosteric interaction of ligand and G proteins (De Lean, Stadel et al. 1980). However, as mentioned in the introduction controversy still exists concerning whether GPCRs are pre-assembled/pre-coupled with heterotrimeric G proteins or if the GPCR–G protein interaction is based on random collision in the membrane (Neubig 1994, Oldham and Hamm 2008). Experimental evidence for all scenarios have been obtained (Hein, Frank et al. 2005, Nobles, Benians et al. 2005, Gales, Van Durm et al. 2006, Qin, Dong et al. 2011). Collision coupling postulates receptor–G proteins interaction only upon receptor activation. Pre-assembly of inactive GPCRs with G proteins, however, more readily explains receptor–G protein selectivity as well as the rapid responses observed in cells (Oldham and Hamm 2008).

Along this line, I would like to make a clear difference between the term pre-coupling and pre-assembly. Unfortunately, these semantic distinctions are not universally applied or understood. Many scientists would not make a distinction between these modes of interaction. Nevertheless, all terms describe, to my understanding, the direct interaction of a G protein with its receptor. Just the degree of interaction might be distinguishable by using the different terms. Pre-coupling in this regard refers to an interaction of the G protein with an activated receptor (agonist activated or constitutive active), in which the G protein is about to become activated.

Pre-assembly or inactive-state-assembly refer to the interaction of an inactive receptor with an inactive G protein. Upon activation of the receptor, one might refer again to a pre-coupling. In general, a full activation of a receptor might look like this:

Inactive-state-assembly/Pre-assembly -> pre-coupling -> coupling -> signaling -> dissociation -> Inactive-state-assemble/pre-assemble

If, how and to what extent DVL plays a role in the context of FZD-G protein interaction has neither been addressed nor even touched.

4.2 BIFUNCTIONAL ROLE OF DISHEVELLED

Even though FZDs consist of seven trans-membrane domains and according to the International Union of Basic and Clinical Pharmacology belong to the group of GPCRs, it is still a matter of debate if FZDs indeed signal through G proteins and if their capability of G protein coupling has any physiological relevance.

The first work (**paper I**) in this thesis solved the longstanding problem of FZD-G protein coupling. A suitable approach to investigate the pre-assembly or inactive-state-assemble of G proteins and GPCRs is the FRAP method (see materials and methods). FRAP permits the determination of whether GPCR and G protein are separated in the membrane or already paired in the inactive state. This method has already been used to investigate the interaction of β-adrenergic receptors (Dorsch, Klotz et al. 2009), and recently the association of inactive M3 acetylcholine receptors and $G\alpha_q$ proteins were detected (Qin, Dong et al. 2011).

By performing dcFRAP experiments, we showed that FZD_6 is in an inactive-state-assembly with $G\alpha_{i1}$ and $G\alpha_q$ proteins.

This interaction could be disturbed by ADP-ribosylation of adenylate cyclase by pertussis toxin (in the case of $G\alpha_{i1}$) and WNT-5A stimulation according to the ternary complex model. In addition, a pathogenic C terminal FZD_6 Arg511Cys mutation known to cause nail dysplasia in human (Frojmark, Schuster et al. 2011) did not show any interaction with $G\alpha_{i1}$ or $G\alpha_q$.

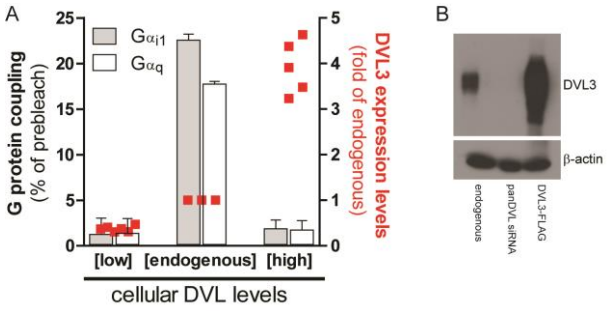


Figure 5 $G\alpha_{i1}$ and $G\alpha_q$ protein coupling efficiency from dcFRAP experiments (left axis) was correlated with DVL3 expression levels (right axis) in panDVL siRNA-treated HEK293 cells, normal HEK293 cells and in cells transfected with DVL3-FLAG (individual dcFRAP data for FZD_6-$G\alpha_{i1}$ and -$G\alpha_q$ coupling with overexpression of DVL1, 2, 3 or panDVL siRNA treatment are presented in **Paper 2**.

In regard to the scaffold protein DVL, both receptors (wt and Arg511Cys) were interacting with DVL when overexpressed assessed by dcFRAP.

To further dissect this newly discovered FZD-G protein interaction, we used cellular imaging in combination with FRAP and Förster resonance energy transfer (FRET) and identified DVL as a regulator of G protein signaling. Modulating DVL levels by either overexpression or siRNA resulted in the loss of $G\alpha_{i1}$ and $G\alpha_q$ inactive-state-assembly to FZD_6 (**Figure 5**). This phenomenon has been further explored in cells expressing endogenous levels of FZDs and the same modulation of DVL levels, functionally impairing WNT-induced and G protein-dependent signaling through P-ERK1/2.

By using different DVL3 deletion constructs we further defined specific domains in this newly discovered dual role of DVL as a master regulator of FZD_6-G protein interaction (**Figure 6**).

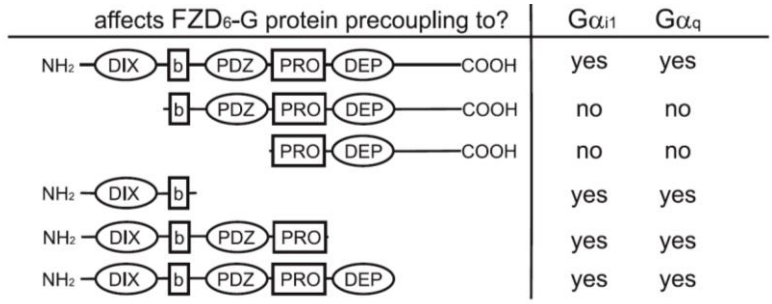


Figure 6 Domain mapping using deletion mutants of FLAG-tagged DVL3 identifies the DIX domain to be important for the negative regulation of FZD-G protein inactive-state-assemble upon overexpression.

Based on our data and what is known from classical GPCRs, we proposed in this study a higher order complex model integrating the function of WNT/FZD/DVL and heterotrimeric G proteins. This complex model was further analyzed in **paper II**. Overall, the results in **paper I** shed light on underlying mechanisms of WNT/FZD signaling specification and add new concepts on the role of heterotrimeric G proteins and the scaffold protein DVL for WNT/FZD signaling. We provided novel insight into previously unappreciated mechanisms with potential impact on the field of biology of WNT-based cellular communication, WNT/FZD pharmacology and WNT/FZD signal transduction. These data could have further importance for the understanding of FZD transduction in embryonic development, cancer and for the future development of FZD-targeting drugs.

We used the same technique to address inactive-state-assembly of FZD_4 and FZD_{10} and G proteins.

In the case of FZD_4, $G\alpha_{12}$ and $G\alpha_{13}$ turned out to be the G proteins able to bind to the receptor whereas FZD_{10} seemed to be exclusively interacting in an inactive-state-assembly fashion with $G\alpha_{13}$ (unpublished data). This also supports the selectivity and sensitivity of the method.

4.3 DYNAMIC DIMERIZATION OF FZD_6

Following up on the results from **Paper I**, we were interested in the stoichiometry underlying the balance between G protein and DVL coupling to FZD_6. In theory, a receptor dimer would be necessary to accommodate the binding proteins (DVL and G protein proposed in **paper I**) due to space limitations. With the help of computational protein modeling using the closely related Smoothened (SMO) structure (Wang, Wu et al. 2013), we were able to model a homodimer of FZD_6, which pointed to a TM4/TM5 dimer interface. The presented dimer model was complemented by dcFRAP and FCCS experiments in combination with site directed mutagenesis to identify a TM4/5 interface homo-dimer of FZD_6.

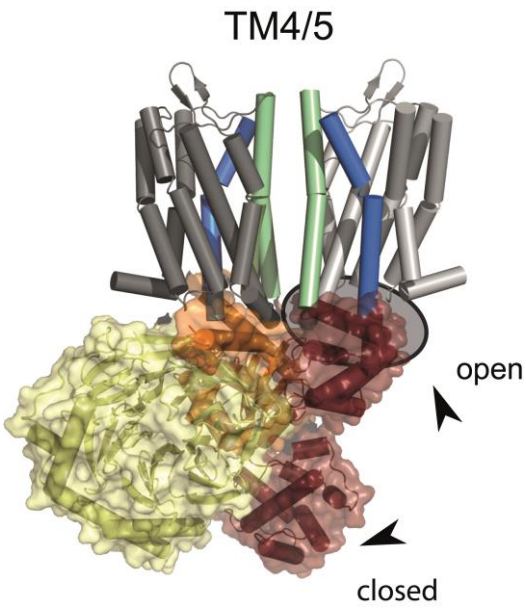


Figure 7 Hypothetical models of a TM4/5 GPCR dimer complex incorporating a G protein (Cordomi, Navarro et al. 2015).

Interestingly, according to a recent publication, this interface is also the one that most likely accomplishes G protein activation compared to dimers with a TM1/TM1, TM5/6 and TM4/4 interface (Cordomi, Navarro et al. 2015). According to the Cordomi et al. model, the open conformation of the α-helical domain of the G protein, observed in the crystal structure of the β_2AR-Gα_s complex, would sterically clash with the second protomer of a TM4/5 dimer (**Figure 7**).

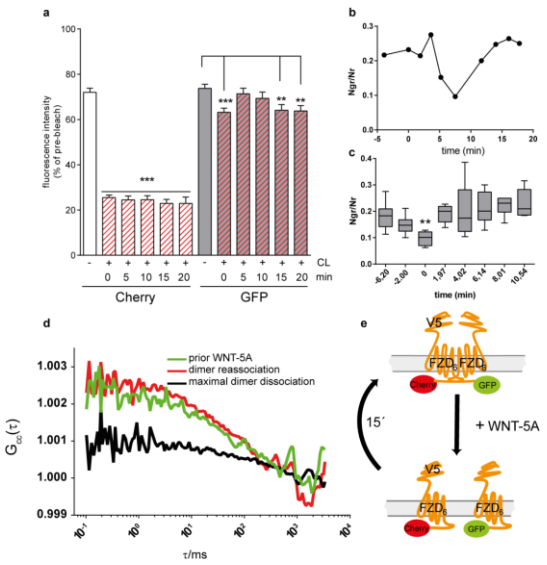


Figure 8 The dcFRAP assay in HEK293 cell expressing V5-FZD_6-Cherry and FZD_6-GFP allowed kinetic monitoring of receptor-receptor interactions upon WNT-5A addition (300 ng/ml). Mobile fractions were measured in the absence of agonist (0 min) and after 5, 10, 15, 20 min of WNT-5A stimulation. The transient increase of the FZD_6-GFP mobile fraction at 5-10 min indicates decreased dimer formation. Error bars give s.e.m.; each bar represents 30-40 ROIs of 8-10 different experiments. (b) Dynamic FCCS experiments were performed to confirm the dynamic and agonist-dependent nature of the FZD_6 dimer. (c) Based on the data we suggest a kinetic model of FZD_6 de- and re-dimerization upon WNT-5A stimulation.

One possible explanation for this would be that the conformation of the α-helical domain observed in the β_2-adrenergic receptors -$G\alpha_s$ crystal might be under physiological conditions smaller in size. Though, in this study we provided another possible mechanism, which has been previously been unrecognized: dynamic, agonist-induced dissociation of FZD_6 dimer.

In the case of FZD_6, agonist (WNT-5A) stimulation led to the receptor dissociation of the dimer and G protein-dependent signaling through P-ERK1/2 was induced (**Figure 8**). This novel concept in GPCR receptor activation characterized by the agonist-induced, transient dissociation and re-association of a GPCR dimer, has not been shown previously (**Figure 9**), provides however, another mode of activation and the possibility to incorporate two FZD_6, a G protein and DVL without the sterically clash as mentioned above.

The bifunctional role of DVL as described in **Paper I** appears to have no influence on the formation of a FZD_6 homo dimer assessed by modulating DVL levels by either overexpression or siRNA.

This suggests that FZD_6 dimerization is rather receptor intrinsic and does not require further binding partners. Next, we related the kinetics of FZD_6 dissociation/re-association with agonist-induced signaling to ERK1/2.

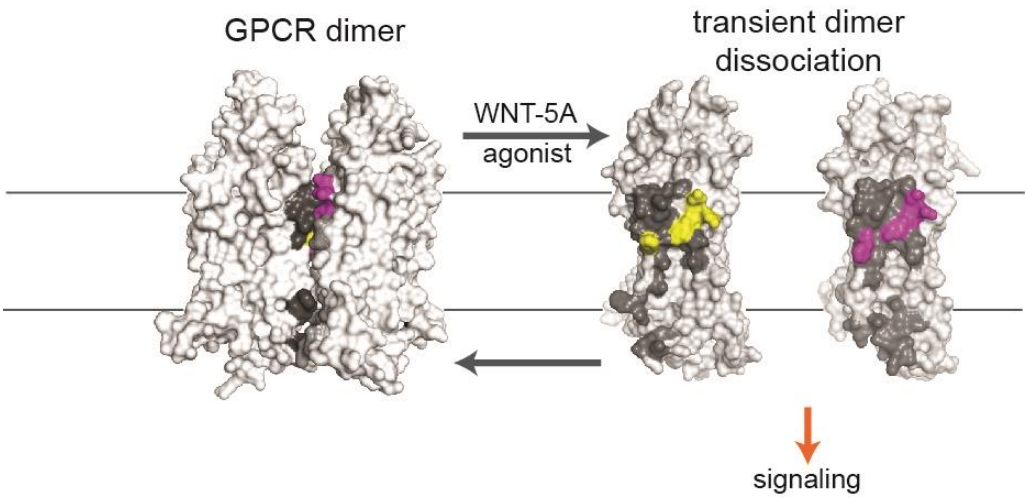


Figure 9: GPCR receptor activation characterized by the agonist-induced, transient dissociation and re-association of a GPCR dimer.

Genome editing of FZD_6 expression level and modification of the proposed dimer using expression of FZD_6 derived TM5 minigenes led to a decrease of basal P-ERK1/2 in mouse lung epithelial (MLE) cells, endogenously expressing FZD_6. However, with respect to the aformentioned minigenes, it is important to mention that our experimental data provide evidence for an inactive state-dimer, which is disrupted by the TM5 minigenes and kept inactive through possibly negative allosteric binding to TM5 peptide.

Furthermore, WNT-5A stimulation of MLE cells led to a time-dependent induction of P-ERK1/2 matching the dynamics in dimerization we obtained with dcFRAP and FCCS supporting the idea of receptor dissociation playing a role in signal initiation. Overall, in this study we proposed a novel mechanism for GPCR activation induced by WNT-5A stimulation of FZD_6.

Our findings shed light on the molecular mechanisms underlying GPCR/FZD complex composition, stoichiometry and dynamics and offer insight into the kinetics of WNT/FZD signal initiation. We further provide evidence for the physiological relevance of FZD_6 dimerization in lung epithelial cells by employing dimer-interfering minigenes in cells that endogenously express FZD_6 (**paper II**). Therefore, targeting the dimer interface to impede with receptor activation in vivo, might offer novel therapeutic possibilities. This study, in combination with **paper III**, provides in addition evidence that the monomeric FZD_6 is inducing signaling and that the interaction of TM5 with monomeric wt FZD_6 maintains an inactive state possibly through action as a negative allosteric modulator (NAM). Therfore, depending on the application, maintaining the dimer will promote a functional inactive receptor complex and interfering with the dimer interface can – depending on the mode of action - promote monomeric (active) FZD_6 or monomeric (inactive) FZD_6 as shown for the TM5 minigenes.

4.4 THE MISSING ELEMENT IN WNT/FZD SIGNALING

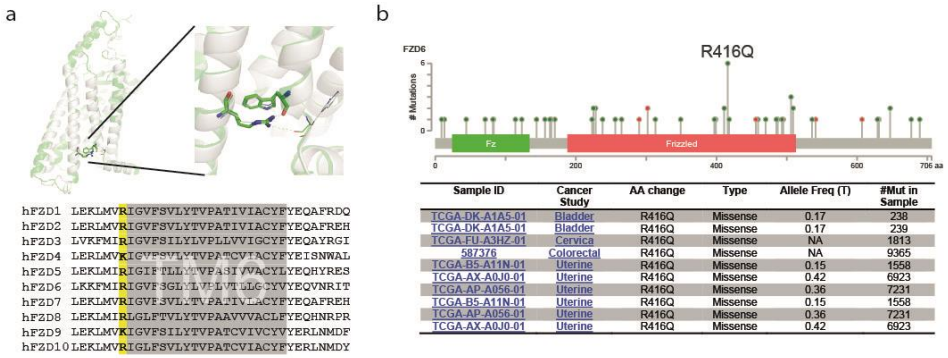
Overall, the identification of the bifunctional role of DVL as crucial and negatively regulating factor for FZD-G protein inactive-state-assembly (**paper I**) together with the underlying dynamics in FZD_6 activation (**paper II**) might explain (i) previous difficulties to mechanistically identify FZD-G protein coupling of FZDs and (ii) the unclear data on the involvement of G proteins and DVL as well as their relative localization to each other in several cellular systems (Liu, Rubin et al. 2005). Nevertheless, claiming that DVL is the missing link between G protein coupling and FZDs would be an oversimplification. It is important to mention that researchers have struggled to characterize FZDs to the same degree as other GPCRs for which structures and robust signaling readouts are available.

One of the more recent publications claims that the DVL-associating protein Daple (homolog of GIV/Girdin) is a guanine nucleotide exchange factor (GEF), which was suggested to serve as the missing factor in FZD signaling through G proteins (Aznar, Midde et al. 2015). While I find the idea of a non-receptor GEF appealing, the study was unable to provide sufficient evidence proving that Daple is as effective in activating heterotrimeric G protein as GPCRs are. However, according to the study Daple is "trapping" DVL to the cytoplasm and competes for FZD binding at the plasma membrane. In regard to the bifunctional role of DVL one might speculate that a delicate balance of DVL, Daple and G protein might be required for full activation of Class Frizzled receptors. Nonetheless, taken the bifunctional role of DVL into account, it seems oversimplifed to generalize this to the entire FZD receptor family, since FZD_4 and FZD_{10} for example, did not show an altered G protein coupling when DVL was knocked down or overexpressed (unpublished data).

4.5 STRUCTURAL ANALYSIS AND DISCOVERY OF A MOLECULAR SWITCH

Molecular modelling of FZD_6, using the recently published high resolution Smoothened structure as a template, identified a potentially interesting residue that could have implications for receptor activation. This residue FZD_6-R416 is located in TM6 and likely forms hydrogen bonds with W493 and W495 located in TM7.

Site directed mutagenesis of this particular residue resulted in a receptor with high internalization dynamics and intrinsic constitutive activity with regard to the positive regulation of G protein-dependent activation of mitogen-activated protein kinases (MAPK) and the inhibition of β-catenin-dependent WNT signaling. Most interestingly, this residue – or an alternative basic residue such as Lys in case of FZD_4 and FZD_9 – is conserved among almost all Class Frizzled



Sample ID	Cancer Study	AA change	Type	Allele Freq (T)	#Mut in Sample
TCGA-DK-A1A5-01	Bladder	R416Q	Missense	0.17	238
TCGA-DK-A1A5-01	Bladder	R416Q	Missense	0.17	239
TCGA-FU-A3HZ-01	Cervica	R416Q	Missense	NA	1813
587376	Colorectal	R416Q	Missense	NA	9365
TCGA-B5-A11N-01	Uterine	R416Q	Missense	0.15	1558
TCGA-AX-A0J0-01	Uterine	R416Q	Missense	0.42	6923
TCGA-AP-A056-01	Uterine	R416Q	Missense	0.36	7231
TCGA-B5-A11N-01	Uterine	R416Q	Missense	0.15	1558
TCGA-AP-A056-01	Uterine	R416Q	Missense	0.36	7231
TCGA-AX-A0J0-01	Uterine	R416Q	Missense	0.42	6923

Figure 10 a) homology model of FZD_6 based on the high resolution crystallographic structure of SMO. $R416^{6.33}$ (IC tip of TM6) is conserved in all human FZD's except FZD_4 & FZD_9 (Lys) b) R416 in FZD_6 is mutated frequently in bladder and uterine cancers.

receptors and mining of available mutational data of different patient-derived tumors revealed mutations in that residue in various FZDs with high frequency in many forms of cancer (**Figure 10**). Detailed analysis of the internalization of FZD_6-R416A argues that the internalized receptor localizes to RAB5-positive endosomes and lysotracker-positive lysosomes. In depth analysis of the constitutive activity of the receptor suggests a central role of heterotrimeric G proteins in signaling to the MAPK pathway and in the inhibition of the WNT/β-catenin pathway. Furthermore, FZD_6-R416A leads to a reduction in basal LRP6 phosphorylation and DVL2 shift as shown by western blotting.

Overall one might speculate that the identified basic residue R presents a general molecular switch among Class Frizzled receptors.

In order to obtain a proof-of-principle and the general importance of the R416A constitutive activity, we extrapolated our findings to FZD_{10} also generating a constitutively active variant of this receptor isoform, negatively regulating β-catenin activation and reducing the DVL shift. The findings in this study provide unique insight into the WNT-induced molecular activation mechanisms of FZDs.

Further, we uncover central and well conserved mechanisms - similar to what is known as the "ionic lock" in Class A GPCRs - that could provide molecular mechanisms for the development of many different forms of cancer. Especially in regard to the frequency R416 is mutated in cancer (**Figure 10**) and the strong internalization phenotype, it could provide a suitable platform for high throughput screening.

So far, we have not succeeded in identifying the factor involved in the observed negative regulatory effect of FZD_6 on β-catenin (**paper III**). Though purely speculative, together with the accumulating evidence that the Hippo pathway might be regulating the WNT/FZD pathway through $G\alpha_{12/13}$ and $G\alpha_q$ (Hergovich and Hemmings 2010, Varelas, Miller et al. 2010, Imajo, Miyatake et al. 2012, Kim and Jho 2014, Wang, Ye et al. 2014, Park, Kim et al. 2015), which in turn is regulating DVL, might give a plausible explanation in regard to the negative regulation of β-catening through FZD_6.

4.6 MONITORING ACTIVITY OF THE WNT/PLANAR CELL POLARITY PATHWAY

So far this thesis has focused mainly on the G protein signaling aspect of WNT/FZD signaling. However, as mentioned earlier, the WNT/FZD pathway is divided into several different signaling branches. Cell polarization is a prerequisite for the control of cell shape, directional migration, asymmetric cell division and function of cells within complex tissues.

One of the key pathways, which control cell polarity, is the WNT/PCP pathway. WNT/PCP controls migration and cell polarity during development of all multicellular organisms from cnidarians to human. The function of the mammalian WNT/PCP machinery is still poorly understood and progress is

hampered mostly by experimental barriers and limitations of non-mammalian experimental models.

A recent report showed that the WNT/PCP pathway is driving the pathogenesis of chronic lymphocytic leukemia (CLL). It was shown that PCP pathway key components such as Vangl2, Celsr1, Prickle1, FZD_3, FZD_7, DVL2, DVL3, and casein kinase 1 (CK1)-ε are upregulated in B lymphocytes of patients with CLL (Kaucka, Plevova et al. 2013). Most interestingly, in this study, it was shown that patient-derived B-cells, expressing to a higher extend FZD_3, FZD_7 and Prickle1, had a less favorable prognosis (Kaucka, Plevova et al. 2013). In the last paper of this thesis (**paper IV**), we used the well characterized CLL cell line MEC1 to develop a useful and powerful model for studying PCP and CLL behavior in vitro, which can easily be manipulated. It is for example, ideal for high throughput screening of chemical compounds targeting mammalian PCP signaling. We demonstrate that several PCP proteins are asymmetrically localized in migrating MEC1 cells (**Figure 11**).

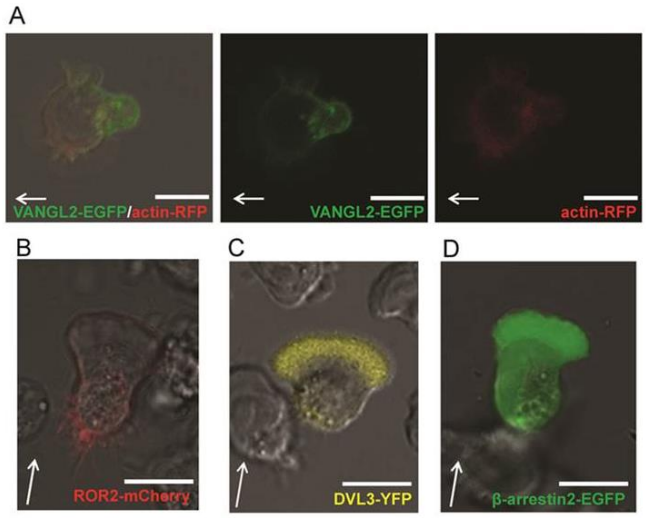


Figure 11 Subcellular localization of fluorescently-tagged PCP proteins in polarized MEC1 cells. (A) Photomicrograph of migrating polarized MEC1 cell with precisely defined leading and trailing edge. Vangl2-EGFP is significantly enriched in the trailing edge of the cell. To visualize the cytoskeleton, actin-RFP was co-transfected with the Vangl2 construct. Arrow indicates the direction of migration. Size bar = 10 µm. (B, C, D) Polarized structure of migrating MEC1 cells expressing ROR2-mCherry, DVL3-EYFP and β-arrestin 2. Arrows indicate the direction of cell migration. Size bars = 10 µm.

This is similar to the localization of PCP protein observed in Drosophila wing or eye where polarized localization of PCP proteins serves as a useful readout of PCP pathway activity (McNeill 2010).

By correlating asymmetric localization of fluorescently-tagged PCP proteins such as Vangl2 with MEC1 cell migration and chemotaxis, we showed that casein kinase 1 maintains the polarized localization of Vangl2 in the trailing edge and at the same time is required for directed cell migration (**paper IV**).

Despite this, the highlight of this story is that for the first time clear polarization of PCP proteins could be observed in vitro in cells that are not embedded in tissue, and lack permanent cell-cell contact.

This work defines a long–awaited mammalian cell-based readout for analysis of activity and asymmetric localization of PCP proteins, which was so far limited to the powerful, but evolutionary distant, Drosophila system. The novel assay developed in this study opens for the first time possibilities to study mammalian PCP signaling in a cell culture assay. Moreover, the assay can be easily automated, which allows the design of screens aimed at identification of novel PCP pathway components and PCP pathway-targeting drugs.

5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Class Frizzled and Class A receptors share many common aspects and at the same time show substantial diversity. They share very similar structural features (**Figure 1**) and all couple to heterotrimeric G proteins (Dijksterhuis, Petersen et al. 2014, Kilander, Petersen et al. 2014). Nevertheless, it is still a matter of debate if Class Frizzled receptors are indeed GPCRs and if a physiological relevance lies behind the observed coupling to heterotrimeric G proteins. However, this thesis provides not only possible new, previously unappreciated mechanisms through which FZDs signal but also novel explanations why it is so diffiult to study this unconventional type of GPCR.

Despite the novel structural and functional aspects of WNT receptors provided in this thesis, the view on FZDs is diversely discussed in the field and multiple pathways and numerous factors supporting or disrupting signaling have been proposed (see introduction). Here, we propose a higher order complex consisting of two FZD_6 incorporating one DVL and a heterotrimeric G protein. This however, needs to be elucidated in more detail to reveal the exact stoichiometry between receptor, DVL and G proteins and the underlying ligand-induced dynamics. Furthermore, we concluded that in the case of FZD_6 a dimeric receptor is required to form a functional inactive receptor complex and, once activated, the monomeric FZD_6 induces signaling. In this context, numerous studies have shown that activation of GPCRs and G proteins does not necessarily require the binding of a ligand to the receptor (Robinson, Cohen et al. 1992, Porter, Hwa et al. 1996, Kenakin 2001, Seifert and Wenzel-Seifert 2002). This is referred to as constitutive activity, where a receptor activates G proteins in the absence of agonists.

Also mutations in the receptor (Alewijnse, Timmerman et al. 2000, Clark, Dever et al. 2004) and truncation of the third intracellular loop & the c-terminus can cause constitutive activity (Boone, Davis et al. 1993, Lefkowitz, Cotecchia et al. 1993, Paavola, Stephenson et al. 2011). Constitutive activity has not been addressed for FZDs yet. In this thesis, we provide the first evidence that FZD_6-wt has intrinsic constitutive activity when overexpressed. A dynamic equilibrium between inactive dimers and potentially active monomers is present (**paper II**) and that mutations in TM6 increase ERK1/2 signaling through FZD_6 (**paper III**). Though highly speculative, FZD_6-R416A could be a biased (constitutive active) mutant. Since we propose a FZD_6-wt dimer consisting of one DVL and one G protein, one might speculate that FZD_6 can activate either DVL or G protein related signaling depending on the activation of the receptor. In this regard, the FZD_6-R416A would be "biased" towards G protein signaling since it has no effect on DVL and induces agonist-independent ERK1/2 signaling. This theory is supported by the fact, that the FZD_6-R416A mutant neither co-localizes with DVL2-GFP nor induces DVL2 shift in HEK293 cells. Furthermore, the negative effect that was observed on LRP6 phosphorylation could be explained by the lack of DVL interaction with FZD_6 which might have important implications for signaling. DVL was shown to be required for LRP6 phosphorylation and aggregation (Bilic, Huang et al. 2007).

In addition, it would be interesting to investigate the mechanisms through which DVL affects stoichiometry of FZD complexes. In other words, why is DVL required for the interaction between FZD_6 and G-proteins, but not a necessary component for FZD_6 dimer formation? Does DVL induce a particular conformational change to achieve G protein binding?

Or is DVL promoting the recruitment of, for example, Daple to act as a GEF? Furthermore, it is unclear if DVL is required for the receptor dynamics observed in **paper II**.

Future studies will offer new insight into Class Frizzled receptors and provide researchers with pharmacological tools to target them. To gain an even deeper and more detailed understanding of FZDs, it would be of great importance to define the smallest functional unit in regard to FZD activation as it was shown for rhodopsin (Tsukamoto, Sinha et al. 2010, Bayburt, Vishnivetskiy et al. 2011). This however, requires purification and reconstitution of FZDs into so called HDL particles. The potential of such particles would be immense. The aforementioned stoichometry can not only be better defined but also the role of DVL, Daple, LRP5/6 and other proteins can be elucidated using GTPγS binding studies. Furthermore, WNT selectivity can be addressed in a new and more physiological fashion, which is so far restricted to CRD binding studies.

Using dcFRAP or the mentioned HDL-particles in combination with FZDs lacking the WNT-binding domain, could also further answer the question if receptor dimerization is a prerequisite for ligand binding and if one WNT is sufficient to activate one or possible both receptors and if this is dependent on different WNT/FZD combination?

From the drug developmental point of view and the connection between impaired WNT signaling and various diseases, FZDs are a very attractive target for drug therapy. There are however, several complicating factors for drug development, most notably the lack of assays to monitor WNT-FZD interaction or direct FZD activation.

The establishment of an HTS assay for FZDs has also been hampered because coupling of a detectable tag to WNTs affects its biological activity, which is important for the binding and activation to FZDs. Another complicating factor is the lack of small molecule agonists or antagonists for FZDs. Development of agonist and antagonist as potential treatment for diseases targeting conditions with downregulated versus overexpressed WNT-signaling is of great significance (Schulte 2010).

The development of selective drugs targeting GPCRs is challenging due to several reasons i) there is a high degree of homology among many closely related receptor subtypes that can regulate diverse physiologic function ii) a single receptor can couple to different G proteins and initiate even G protein-independent pathways iii) a receptor can allosterically be influenced by receptor homo-dimers or hetero-dimers and iv) signaling behavior of GPCRs might differ dependent on different cells or organs (Trzaskowski, Latek et al. 2012).

The improved understanding of signal transduction initiated by FZDs provided in this thesis could reveal new mechanisms suitable as targets for new drugs. Furthermore, the techniques employed in **papers I, II** and **IV** could open up new avenues for high throughput screening assays for the development of small molecules targeting WNT receptors.

In summary, the functional studies described in this thesis are mostly incompatible with the classic concept of the two-state equilibrium model of GPCR activation.

A multistate model would be more appropriate, where ligands can stabilize specific conformations or subsets of activities (Deupi and Kobilka 2010) making the complexity of GPCR signaling similar to the microprocessor work[4].

[4] Kenakin T. In: GPCR Molecular Pharmacology and Drug Targeting. Shifting Paradigms and New Directions. Gilchrist A, editor. John Wiley & Sons: John Wiley Sons. Hoboken; 2010. pp. 1–26.

6 CONCLUSION

Paper I

- FZD_6 can couple to $G\alpha_{i1}$ and $G\alpha_q$
- WNT-5A stimulation leads to the dissociation of $G\alpha_{i1}$ and $G\alpha_q$ in a time dependent manner
- A pathogenic C terminal FZD_6 Arg511Cys mutation known to cause nail dysplasia in human does not interact with $G\alpha_{i1}$ or $G\alpha_q$.
- Inactive-state-assembly is dependent on a delicate balance of DVL

Paper II

- FZD_6 dimerizes through a TM4/5 interface
- Agonist stimulation results in the transient dissociation of the FZD_6 dimer
- Dimeric FZD_6 is inactive, wheras upon activation FZD_6 becomes monomeric and initiates signaling
- Kinetics of FZD_6 dissociation/re-association coincide with agonist-induced signaling to ERK1/2
- Genome editing and expression of FZD_6-derived TM5 minigenes interfered with FZD_6-dependent signaling to ERK1/2 in lung epithelial cells

Paper III

- Identification of a molecular switch in TM6/7 of FZD_6
- The FZD_6-R416A mutant (molecular switch) negatively regulates the β-catenin pathway and activates G protein-dependent ERK1/2 signaling
- $G\alpha_q$ activation appears to be relevant for the inhibitory effect of β-catenin
- R416 in FZD_6 is frequently mutated in bladder and uterine cancer
- The molecular switch seems to be a general feature of activation in Class FZD receptors

Paper IV

- MEC1 cells are a suitable screening model for chronic lymphocytic leukemia
- Clear polarization of fluorecently tagged PCP proteins observed in a unicellular context
- PCP proteins, such as Vangl2-EGFP, serves as a useful readout of PCP pathway activity
- Casein kinase 1 maintains polarized localization of Vangl2 in the trailing edge and at the same time is required for directed cell migration
- Polarization of Vangl2 is independent of an intact actin cytoskeleton

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